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Original article

Effects of ingestion of a biotin-binding protein on adult and larval honey bees

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Abstract – The insecticidal properties of biotin-binding proteins (BBPs) have recently been exploited in transgenic plants. As BBPs have a broad spectrum of insect toxicity, their potential impacts on non-target insects such as honey bees need to be assessed. In this study, the effects of feeding a purified BBP, avidin, to honey bee larvae and adults were determined. A realistic larval dosing regime was developed by estimating the pollen content of brood food in the field and adding avidin to artificial diet at rates that simulated the presence of avidin-expressing transgenic pollen in brood food. Larval survival and development were unaffected by avidin in assays which simulated larvae receiving pollen expressing 0, 4 or 40 μM avidin at concentrations of 164 μg pollen per mg food for the first 2 days and 880 μg pollen per mg food thereafter. Food consumption and survival of adult bees were also unaffected by avidin added to pollen-candy at levels corresponding to pollen expression of 0, 6.7 or 20 μM avidin.

Apis mellifera **/ biotin-binding protein / avidin / transgenic plant**

1. INTRODUCTION

The biosafety of insect-resistant transgenic plants to beneficial insects such as honey bees has been the subject of an increasing number of studies in recent years (Malone and Pham-Delègue, 2001). So far most investigations have focused on plants expressing *Bacillus thuringiensis* (Bt) toxins or proteinase inhibitors (PIs). Less is known of the impacts of other pest-resistant transgenic plants or transgene products on bees.

Recently, two different strategies have been developed for producing transgenic plants expressing biotin-binding proteins (BBPs) and this has renewed interest in these insecticidal proteins. One strategy targets expression to the vacuoles (Christeller et al., 1999; Murray et al., 2002) and the other to cell walls (Hood et al., 1997; Kramer et al., 2000). As their name suggests, the BBPs are thought to act by binding with dietary biotin and creating a lethal deficiency of this vitamin in susceptible insect species. Trials in which insect larvae have been fed

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with purified BBPs, such as avidin (originally isolated from chicken egg white) and streptavidin (from the bacterium *Streptomyces avidinii*), have shown significant growth suppression and mortality in representatives of the Lepidoptera, Coleoptera and Diptera (Morgan et al., 1993; Markwick et al., 2001). Transgenic tobacco plants expressing 2 to 20 μM vacuole-targeted avidin have been shown to stunt and kill larvae of the cotton bollworm, *Helicoverpa armigera*, and the common cutworm, *Spodoptera litura* (Burgess et al., 2002). Similarly, transgenic maize kernels expressing 20 to 2 500 ppm (1.3 to 161 μM) cell-wall-targeted avidin have been shown to be insecticidal to pests of stored products (Kramer et al., 2000).

Bees could be exposed to insecticidal proteins from transgenic plants if there is significant transgene expression in the pollen, nectar or resin of these plants. Nectar is composed primarily of sugars and recent investigations in leek and tobacco have revealed a total protein content of only 0.02% (w:v) (Peumans et al., 1997; Carter et al., 1999). There are no published reports of protein measurements for plant resins which bees collect for propolis manufacture. Propolis is composed mostly of flavonoids, but one study has demonstrated that it may contain between 2.1 and 3.8% protein (Kaczmarek and Debowski, 1983). Pollen is perhaps the most likely route for bee exposure to transgene products as it is composed of between 7.5 and 35% protein (Schmidt and Buchmann, 1992). The expression levels of BBPs in the pollen of transgenic BBP-tobacco or BBP-maize have not yet been reported, but since pollen has both a cell wall and a vacuole the possibility of such expression exists and theoretically bees could be exposed to BBPs via this route.

Pollen is a significant component of the diets of both larval and adult bees. It has been estimated that colonies require between 20 and 30 kg of pollen per year (Haydak, 1935). Adult bees ingest significant quantities of bee-processed pollen (bee bread) for the first 8 to 10 days of adult life (Haydak, 1970) and the protein thus obtained is necessary for full development of the hypopharyngeal glands and other organs (Crailsheim and Stolberg, 1989). Newly-emerged adult worker bees kept in cages for eight days and supplied ad lib. with an artificial diet containing pollen will each consume 7.1 mg of diet, containing 2.4 mg of pollen, per day (Malone et al., 2001). Larvae in the hive are fed with a mixture of pollen and glandular secretions from adult nurse bees (Haydak, 1943). Older larvae apparently receive progressively more pollen, but figures for individual pollen consumption rates in larvae are scarce. Planta (1888, quoted in Haydak, 1943) noted that the food of older drone larvae contained pollen at a concentration of 15 000 grains per mg. Thus both older larvae and newly-emerged adult bees could potentially be exposed to transgene products expressed in pollen. If we assume that transgenic plants will express BBPs in pollen at similar concentrations to those reported for leaves, we can estimate the amounts of a BBP that an adult bee may ingest, based on what is known about pollen ingestion by these bees. With more information on consumption of pollen by bee larvae, a similar estimate could be made for this life stage.

Since BBPs exert their insecticidal effects by causing a biotin deficiency, their impact will depend not only upon an insect's biotin requirement but also the levels of dietary biotin it receives relative to the concentration of BBP consumed. Honey bee requirements for biotin are unknown (Herbert, 1992). Average levels of 0.32 μg biotin per g of pollen (1.31 μM biotin) and 1.5 μg per g of harvested royal jelly (6.14 μM biotin) have been recorded (Schmidt and Buchmann, 1992). However, biotin levels in bee bread and in the mix of glandular secretions and pollen fed to larvae are unknown. Since many micro-organisms can biosynthesise biotin, it is possible that bee gut microflora and/or micro-organisms present in bee bread may supply this vitamin.

The aim of the present study was to estimate whether the BBP avidin, if expressed in the pollen of a transgenic plant, would have an impact on honey bees. Firstly, we determined the concentrations of pollen grains ingested by bee larvae in field hives, and the weights of some pollen grains, in order to estimate a realistic dosing regime for feeding avidin to larvae in a laboratory-based assay system. Secondly, to

ensure that this system presented the bees with dietary biotin at a realistic concentration, we measured the levels of this vitamin in samples of larval food from beehives and from the artificial diet used in the assay. Biotin levels in bee bread and in bee-collected pollen were also measured to ensure that the pollen-candy mix used in our adult assay presented these bees with realistic levels of this vitamin. Thirdly, bee larvae were fed continuously with avidin in artificial diet at concentrations corresponding to two levels of avidin expression in pollen and the effects on their development and survival were determined. Finally, the effects of feeding newly-emerged adult bees with avidin at two concentrations in a pollen-candy food were determined.

2. MATERIALS AND METHODS

2.1. Counting pollen grains in larval bee food

Frames containing brood were taken from three different colonies of Italian race honey bees (*Apis mellifera* L.) kept in our apiary at Mt Albert Research Centre, Auckland, New Zealand, and brought into the laboratory. About 20 larvae of various ages were removed from their cells and their lengths and weights determined. At the same time, samples of their food were removed, and each was weighed, diluted by the addition of 20 µl of water and mixed thoroughly. A 5 µl aliquot was taken from each diluted sample and placed under an 18 × 18 mm coverslip on a glass microscope slide with a 6 × 6 mm square etched onto it. The number of pollen grains in the etched square were counted and the concentration of pollen in each food sample (grains per mg food) calculated.

2.2. Determining weights of individual pollen grains

In order to establish realistic levels at which to add purified avidin to the artificial diet in our bee larval assay, we determined the weights of a range of pollen grains and converted the concentrations of pollen grains we had found in larval food in the hive to weight: weight ratios. To this end, four samples of mixed-floral bee-collected pollen, which had been stored frozen for about one year, and one sample of fresh tobacco pollen were weighed, mixed thoroughly with 1 ml of water and the concentration of pollen grains in each counted using a haemacytometer. The weight of a single pollen grain was then estimated by dividing the weight of each sample by the total number of pollen grains in that sample.

2.3. Measuring biotin levels in bee foods

Six samples of brood food, each weighing about 10 mg, were collected from three different colonies. One sample from each colony was taken from cells containing larvae weighing about 1 to 5 mg ("younger larvae") and the other from larvae weighing about 10 to 90 mg ("older larvae"). Three samples of bee bread were also taken from hives in the same apiary. The concentrations of biotin in these samples, in three samples of cold-stored, mixed-floral pollen (as above), and three samples of artificial larval diet (Peng et al., 1992) were determined as described by Christeller and Phung (1998). Samples were digested in 6 volumes of 2 M H_2SO_4 for 2 h at 120 ^oC. Samples were diluted 20 fold, neutralised and assayed by competition ELISA. Biotin samples (10–100 µL) and standards (0.1–0.3 ng biotin) were incubated in 96-well immunoassay plates previously coated with avidin (62.5 ng/well), washed and re-incubated with alkaline phosphatase-biotin (25 ng/well) (Sigma Chemical Co.). Concentrations of biotin in the samples were determined from standard curves based on the initial reaction rates at 410 nm following addition of p-nitrophenylphosphate.

2.4. Larval bee/avidin assay

The larval assay method of Peng et al. (1992) was modified and used to determine the impacts of two different concentrations of purified avidin on bee development and survival. One treatment delivered avidin to the larvae at a rate corresponding to exposure to pollen expressing 4 µM avidin. The second treatment delivered ten times that amount (i.e. equivalent to 40 µM avidin expression in pollen). Controls received the same artificial diet with no additive.

The larval bee artificial diet comprised 4.2 g royal jelly powder, 0.6 g glucose, 0.6 g fructose, 0.2 g Difco yeast extract, and 14.4 g distilled, sterilised water (Peng et al., 1992).

Using information obtained above on the concentrations of pollen in brood food in the hive, a two-step regime was devised for both avidin treatments. This simulated a situation where bees received 4 100 pollen grains (each weighing 0.04 µg) per mg food for the first two days of larval life and 22 000 of these grains per mg food for the rest of their larval development. Purified avidin was added to artificial diet at concentrations corresponding to the presence of pollen as above, expressing 0, 4 or 40 µM avidin. Thus for the first two days of the experiment, larvae

received either 0.384 ng ("4 μM in pollen" - treatment) or 3.84 ng ("40 μM in pollen" treatment) avidin per mg artificial diet. From day 3 onwards, they received either 8.56 ng ("4 μM in pollen") or 85.6 ng ("40 μM in pollen") avidin per mg artificial diet. Control larvae were given diet without additive throughout. Larvae were moved to fresh diet each day and a new batch of each diet was made up every three days. Eight replicates of the controls, five replicates of the 4 μM avidin treatment and four replicates of the 40 μM avidin treatment were carried out. There were 30 bee larvae in each replicate.

Purified lyophilised avidin from egg white (Lot 276992) was obtained from the Calbiochem-Novabiochem Corporation (La Jolla, CA 92039). For the diets delivering low concentrations of avidin, appropriate volumes of a stock avidin solution (0.1 mg/ml in water) were added to batches of diet which lacked the equivalent volumes of water. For diets delivering higher avidin concentrations, crystalline avidin was added to the diet. All diets were mixed thoroughly before use.

First instar bee larvae were obtained from Italian race colonies kept at our Mt Albert Research Centre apiary by bringing brood frames into the laboratory and gently lifting out the smallest larvae with a sable brush (size 00). These were then placed, 10 per well, on the surface of the artificial diet (300 μl per well) in 24-well tissue culture plates. Larvae were moved to fresh diet in new wells each day and given progressively more room to grow by placing them five, three, two and then one larva per well over consecutive days. The tissue-culture plates were kept on racks in a desiccator with a super saturated solution of K_2SO_4 in its base (to create an environment with 95% relative humidity, Sweetman, 1933) at 35 ^oC. At the first sign of defaecation (yellow colour in the diet), which occurs just prior to pupation, larvae were gently transferred to clean wells lined with two layers of Kimwipes® tissue paper and placed in a second desiccator containing a supersaturated solution of NaCl (75% relative humidity, Buxton and Mellanby, 1934) at 35 ^oC. Prepupae and pupae were checked daily. When close to adult emergence, each pupa was provided with a small quantity of bee bread and sugar candy, so that the newly-emerged adult bee could obtain some food immediately. At this time, the tissue-culture plates were also placed individually inside mesh bags sealed with Velcro®, to ensure that adults emerging from different treatments did not become mixed. Each day new adults were transferred in groups to small wooden cages, one cage per treatment, supplied with water, sugar syrup and protein food (0.33 parts pollen, 0.08 parts sodium caseinate, 0.16 parts brewer's yeast and 0.43 parts sucrose mixed with water to a paste) and kept in darkness at 33 ^oC until all had died.

The percentages of bees surviving to defaecation, to adulthood and from defaecation to adult emergence were transformed by taking arcsine square roots and compared by ANOVA (Payne et al., 1993). ANOVA was also used to compare mean numbers of days until defaecation and until emergence. Median and mean longevities of all bees in the experiment and of those which survived to adulthood were compared using χ^2 tests and ANOVA, respectively.

2.5. Adult bee/avidin assay

To assess the impacts of avidin on adult bees, we presented newly-emerged adult bees with a pollen-candy mixture to which avidin had been added at one of two different concentrations. The method used was similar to that described in Malone et al. (1999) for testing a Bt toxin and a PI. Young adult honey bees were collected as they emerged from frames taken from hives at our Mt Albert Research Centre apiary. Bees were assigned randomly to cages (30 bees per cage), supplied with water and sugar syrup (60% w:v sucrose solution) via gravity feeders, and kept in darkness at 33 ^oC. Each cage also contained a small cup holding a mixture of bee-collected, mixed-floral pollen (as above) (1 part) and sugar candy (2 parts) (candy recipe: Ambrose, 1992) to which avidin (Calbiochem®, Lot 276992) had been added. One group of caged bees received food containing 0.1 mg avidin per g of pollen, which is equivalent to pollen expressing 6.7 μM avidin. The second group of bees received food containing 0.3 mg avidin per g of pollen, which is equivalent to an expression level of 20 μM, the highest level we have encountered in transgenic tobacco leaves (Burgess et al., 2002; Murray et al., 2002). A third group of bees (controls) received pollen/candy without additive. The cages were checked daily for dead bees until all had died. This trial was replicated four times. Median and mean longevities were compared using a χ^2 test and ANOVA, respectively.

To measure consumption of the pollen-candy food by the bees, each cup of food was weighed at the start of the experiment and again at day 8. A new cup with the same type of food was then weighed, placed in each cage and left until day 14 when it was weighed once again. Food consumption (mg per bee) over two periods (days 0 to 8 and days 9 to 14) was estimated by dividing the weight loss from the cup by the number of bees alive on days 8 and 14. Mean food consumption figures were compared by ANOVA.

3. RESULTS

3.1. Pollen grains in larval bee food

Larval bee food (brood food) sampled from hives contained variable amounts of pollen, with that taken from cells containing large larvae tending to contain more pollen than that taken from cells of small larvae (Fig. 1). The mean concentration of pollen in all samples was 2 895 ± 541 pollen grains per mg of food and the range was 0 to 21 969 pollen grains per mg. The frequency distribution of larval weights (data not shown) suggested that five bee instars were sampled. Their approximate weight ranges were 0.2 to 20 mg for the first instar, 21 to 45 mg for the second, 60 to 110 mg for the third, 120 to 150 mg for the fourth and 165 to 185 mg for the fifth. Larval weight was a better indicator of larval size (and thus age) than larval length, since larvae weighing between 37.78 and 185.52 mg were all of similar length (10 mm) (data not shown). Many larvae weighing less than 50 mg (probably first and second instars) had no detectable pollen in their food and none had more than 4 102 pollen grains per mg food (Fig. 1). Larvae weighing more than 50 mg had between 0 and 21 969 pollen grains per mg food.

3.2. Pollen grain weights

The mean weight of a pollen grain in the mixed-floral, bee-collected pollen samples (which had been stored frozen) was 0.021 ± 0.003 μg with a range of 0.008 to 0.039 μg. The weight of a freshly collected tobacco pollen grain was estimated to be 0.011 μg.

3.3. Biotin levels in bee foods

The mean concentrations of biotin in samples of larval bee foods were as follows: 3.45 ± 0.73 μM biotin for brood food taken from cells containing younger larvae, 3.14 ± 0.72 μM biotin for brood food taken from older larvae, and 2.49 ± 0.49 μM biotin for artificial larval bee diet. ANOVA showed that there were no significant differences among these values. Mixed-floral, bee-collected, cold-stored pollen had 1.85 ± 0.08 μM biotin and bee bread had 1.83 ± 0.36 μM biotin.

3.4. Avidin does not affect larval bee development or survival

The addition of purified avidin to artificial diet given to bee larvae, at levels equivalent to that which may be delivered in brood food via pollen expressing 4 or 40 μM avidin, had no impact on the percentage of bee larvae that survived to adulthood, the percentage that survived until defaecation, or the percentage of successful defaecators that survived to adulthood (ANOVA, Tab. I).

The onset of defaecation prior to pupation and of adult emergence was not significantly altered by feeding bee larvae with avidin (Fig. 2). On average, bees fed avidin at a rate equivalent to pollen expressing 4 μM

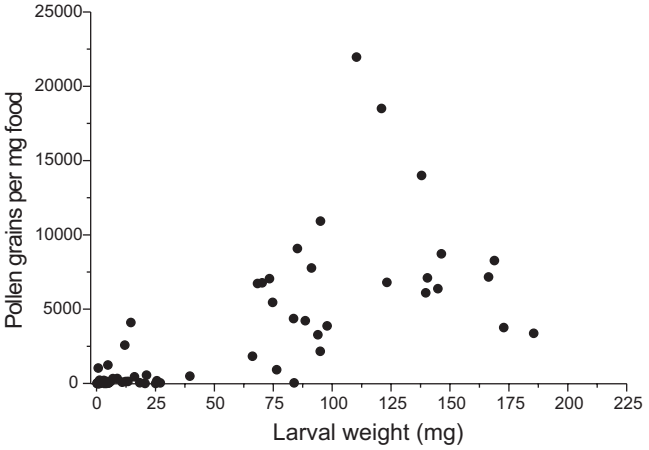

Figure 1. Counts of pollen grains in brood food taken from cells containing bee larvae in field hives plotted against the weight of each larva.

Table I. Mean percentages of bee larvae surviving to defaecation and adulthood.

Treatment	Control	"Low avidin"[a]	"High avidin"[a]
% surviving to defaecation	75.0	87.6	80.0
% surviving to adulthood	48.4	53.0	45.9
% of successful defaecators surviving to adulthood	62.1	59.5	55.9

[a] See "Materials and Methods" for details of avidin treatments.

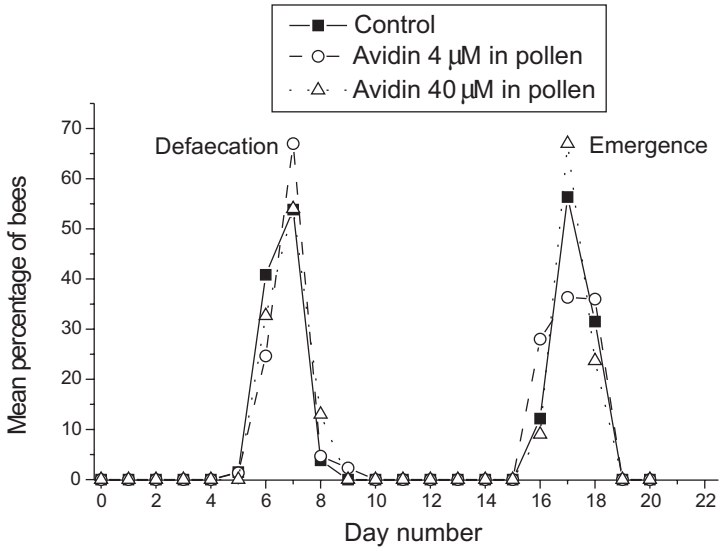

Figure 2. Comparison of the onset of defaecation and adult emergence in days from the beginning of the experiment for bee larvae fed with avidin in artificial diet at concentrations equivalent to those expected in brood food containing pollen expressing 4 or 40 µM avidin, and controls fed diet without additive.

defaecated 6.8 days from the beginning of the experiment, those fed avidin at a rate equivalent to pollen expressing 40 µM defaecated after 6.9 days, and the controls after 6.8 days. There were no significant differences among these values (ANOVA, LSD (0.05) = 0.51). Bees fed the lower concentration of avidin emerged as adults 17.3 days from the start of the experiment, those fed the higher concentration emerged after 17.1 days, and the controls after 17.3 days. There were no significant differences among these values (ANOVA, LSD (0.05) = 0.62).

Larval survival declined steadily over time until 18 days after the beginning of the experiment when there was an abrupt drop in survival for each group of bees (Fig. 3). This corresponded to the first 24 hours following emergence of most bees as adults. Subsequent adult survival was variable, but declined steadily in all groups until all bees were dead (Fig. 3). Comparisons of median and mean longevity of each of the three groups of bees showed that there were no significant survival differences attributable to avidin. Median longevity was 18 days for each group of bees (no significant difference, χ^2 test). Mean longevity was 16.74 days for bees fed avidin at a rate equivalent to pollen expressing 4 µM, 18.47 for bees fed avidin at a rate equivalent to pollen expressing 40 µM and 17.12 days for the controls (no significant difference, ANOVA, LSD (0.05) = 5.754). However, bee survival and longevity were generally very variable and there were significant differences among the different replicates within each treatment.

When the survival of emergent adults only was considered, there was a significant treatment effect on median longevity (χ^2 test, $P = 0.0006$). Bees fed the higher concentration

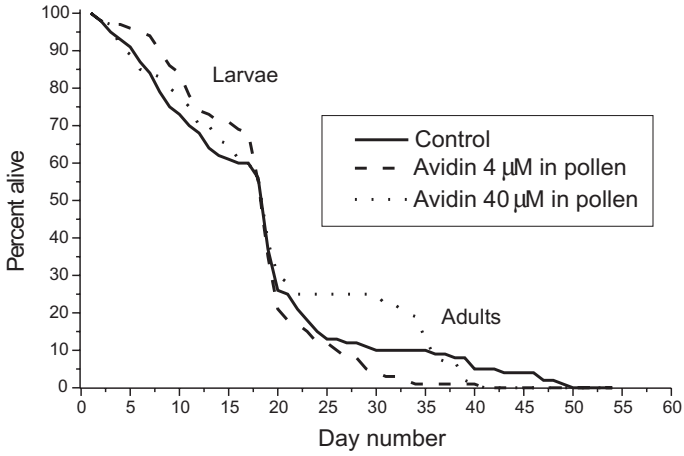

Figure 3. Survival of bees fed throughout the larval phase with avidin in artificial diet at concentrations equivalent to those expected in brood food containing pollen expressing 4 or 40 μM avidin, and controls fed diet without additive.

of avidin as larvae appeared to have had better survival than those fed the lower avidin concentration or the controls (Fig. 3). However, this significant difference was lost when mean longevity was compared by ANOVA (this, unlike the χ^2 test, allows for pairwise comparisons). The mean longevity of successfully emerged adult bees was 6.93 days for bees fed the lower concentration of avidin as larvae, 13.50 days for those fed the higher concentration, and 10.00 days for the controls (ANOVA, LSD (0.05) = 7.877). Once again, there were highly significant differences among replicates within each treatment.

3.5. Avidin does not affect adult bee survival or food consumption rates

Newly-emerged adult bees readily consumed pollen-candy to which purified avidin had been added (Fig. 4). Over the first eight days of exposure to the foods, control bees consumed an average of 99.5 mg of food per bee, those fed the lower concentration of avidin consumed 99.2 mg and those fed the higher avidin concentration consumed 99.7 mg. These figures did not differ significantly from each other (ANOVA, LSD (0.05) = 10.45). Between days 9 and 14, mean consumption in each

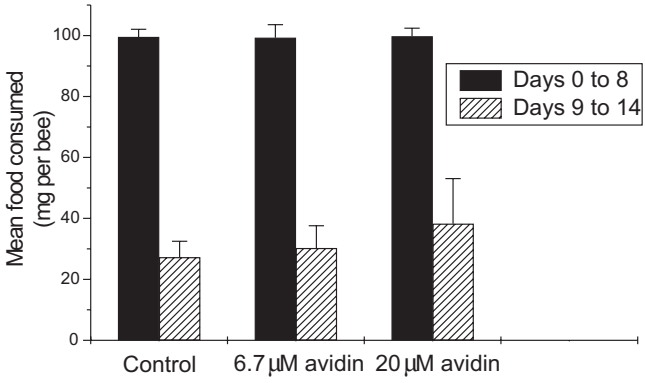

Figure 4. Mean food consumption by adult bees fed with pollen-candy containing avidin at concentrations equivalent to those expected if the pollen was expressing 6.7 or 20 μM avidin. Control bees were fed pollen-candy without additive. The experiment began when the bees were newly-emerged (day 0).

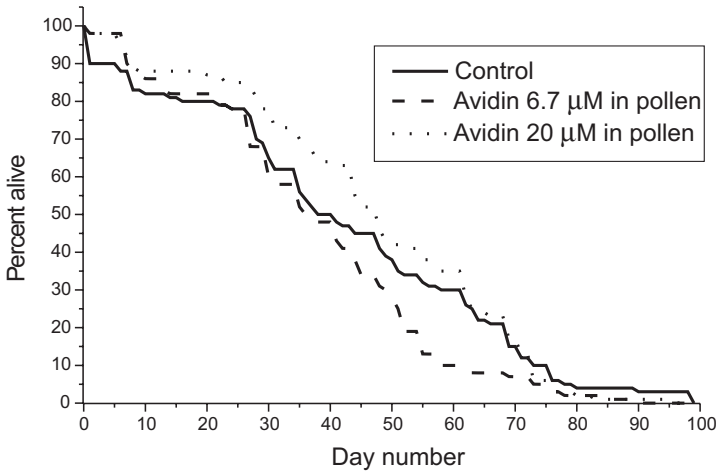

Figure 5. Survival of adult bees fed from emergence with pollen-candy containing avidin at concentrations equivalent to those expected if the pollen was expressing 6.7 or 20 μM avidin. Control bees were fed pollen-candy without additive.

treatment was 27.3, 30.3 and 38.2 mg per bee respectively. Once again, there were no significant differences among these figures that could be attributed to the addition of avidin to the food (ANOVA, LSD (0.05) = 22.91).

There was a significant treatment effect on adult bee survival (χ^2 test, $P = 0.0025$) (Fig. 5), with bees fed the higher concentration of avidin having better median survival (48 days) than those fed the lower avidin concentration (36 days). Control bees had an intermediate median survival time (41 days). There were also significant differences in median survival among replicates within each treatment. However, when pairwise comparisons of mean longevities were made using ANOVA, there were no significant differences among the three treatments (47 days for high avidin, 37.6 days for low avidin, 42.1 days for controls) (ANOVA, LSD (0.05) = 10.858).

4. DISCUSSION

The concentrations of pollen in brood food taken from bee larvae of various ages were similar in magnitude to that noted for a drone larva by Planta (1888) (15 000 grains per mg). As our apiary was located in a suburban area, the bees in our study had access to a variety of garden flowers for forage. It would be interesting to obtain pollen content figures from brood food of bees kept in other situations, especially near agricultural crops.

To establish our dosing regime for feeding a purified transgene product to bee larvae, we converted the counts of pollen grains in brood food to weight: weight ratios. To do this we estimated the range of weights of individual pollen grains from some samples of cold-stored, mixed-floral pollen and of fresh tobacco pollen. This approach had some obvious shortcomings. Firstly, the cold-stored pollen may have dried out and lost weight. Secondly, we measured fresh pollen of only one plant species, tobacco. Despite this, the weights obtained accord reasonably well with previously published estimates, which range from 0.8 ng for *Betula verrucosa* to 1.068 μg for *Cucurbita pepo*, with a median size of 0.0315 μg (Stanley and Linskens, 1974). Thus, the pollen grain weight used for our dosing regime (0.04 μg) was not unrealistic. For further studies focusing on particular transgenic crops, more accurate estimates using the pollen of the appropriate plant species could be made.

Brood food taken from young and old larvae in hives contained about 3 μM biotin. This is

somewhat higher than the biotin concentrations of bee-collected pollen, which ranges from 0.66 to 2.46 μM (present study; Schmidt and Buchmann, 1992), and bee bread (1.83 μM, present study). It seems likely that the secretions from workers' hypopharyngeal glands add biotin to larval bee food, especially since the food received by young larvae contained significant quantities of this vitamin but little pollen. High levels of biotin (6.14 μM) recorded for harvested royal jelly (Schmidt and Buchmann, 1992) lend support to this idea. The artificial diet used in the larval bee assay (Peng et al., 1992) had levels of biotin similar to those found in natural larval bee food and the mixed-floral pollen used in the adult bee assay had a biotin content similar to that of bee bread in the hive. This suggests that the effects of avidin observed here may also be realistically expected under field conditions.

The biotin concentration data presented here suggest that this vitamin is a significant component of the diet of larval and young adult bees. However, the assay data show that ingestion of avidin by these bee life stages, at concentrations to which bees might be exposed when foraging on transgenic plants expressing avidin in their pollen, does not produce a lethal deficiency of this vitamin in these insects. Perhaps bees commonly ingest biotin well in excess of their needs. A second possibility is that their gut microflora synthesise this vitamin, as has been suggested for pantothenic acid (Haydak and Vivino, 1950). A third, perhaps least likely, explanation is that honey bees may have a particularly low requirement for biotin. Herbert and Shimanuki (1978) have shown that vitamins D and K are not essential for brood rearing by bees.

There are fewer published studies of the impacts of pest-resistance transgene products on honey bee larvae than of impacts on adults. Not surprisingly, Bt toxins with no known toxicity for hymenopterans do not appear to affect larval honey bees. Arpaia (1996) fed purified Cry3B (CryIIIB, coleopteran-active) Bt toxin in sugar syrup to bee colonies for two months and found no effects on larval survival or pupal dry weight. Larval toxicity tests of the Bt toxins Cry1Ab, Cry9C (both lepidopteran-active) and Cry3A (coleopteran-active) conducted for EPA registration of Bt-transgenic plants in the USA showed no adverse effects (Anon, 2000). In contrast, Kunitz soybean trypsin inhibitor fed to honey bee larvae at a "high but realistic" concentration increased larval mortality, increased larval and pupal development time, and decreased adult body mass (Brødsgaard et al., 2001). Adult honey bees use trypsin to digest protein and are negatively affected by ingestion of high levels of trypsin inhibitors (Burgess et al., 1996; Malone et al., 1998). It may be that bee larvae use similar digestive proteases. The present results with avidin suggest that bee larvae will not be negatively affected by plants expressing this pest-resistance protein in pollen.

While the laboratory-based larval assay used here and in Brødsgaard et al.'s (2001) study provides a convenient method for testing transgene products, improvements to reduce the levels of control bee mortality (Tab. I) would be desirable. Peng et al. (1992) reported 90.5% survival to defaecation and 81.9% subsequent survival to adult emergence with this method, suggesting that more practice and perhaps gentler handling could bring about such an improvement. The possibility that latent virus infections are activated during the assay and may account for some of the mortality observed also needs to be investigated. A field-based technique used for Cry 1Ac testing, in which droplets of sucrose solution containing the Bt toxin were added to the cells of young bee larvae in hives (Anon, 2000), resulted in control bee survival levels (84% to pupation and 95% to emergence) that were comparable to those of Peng et al. (1992). Thus, in skilled hands, the laboratory-based assay can produce survival rates similar to those attained in the hive.

Interestingly, greater adult bee longevity was noted among bees fed with higher concentrations of avidin than with lower concentrations in the two assays described here. This survival effect was significant both when bees received avidin throughout larval life (but not as adults) and also when they received it from emergence as adults (but not as larvae). Caution is required when interpreting this result however, since significant differences in bee longevity also occurred among replicates within each treatment. Clearly, the assay

methods used need to be improved to reduce unexplained variability in bee survival. Even so, we can conclude that the results obtained thus far do not give cause for alarm over potential bee impacts from the use of avidin-expression technology for pest protection in crop plants.

A number of other areas need to be investigated in order to fully assess the safety to bees of transgenic pest-resistant plants expressing BBPs, Bt toxins, PIs or other transgene products. Effects on queen and drone bees need to be ascertained, as do possible sub-lethal impacts on worker bees. In particular, sub-lethal effects that may affect colony performance, such as those which may interfere with hypopharyngeal gland development, should be quantified. The potential for accumulation of transgene products in food stores in the hive needs to be determined. Coloured sugar solutions containing a PI have been fed to bee colonies to track storage patterns in the hive (Pham-Delègue, personal communication). This method could also be used with other proteins.

The larval and adult testing regimes used here have both assumed that pollen will be the major vehicle for honey bee exposure to avidin. Quantitative data on pollen expression levels for this transgene (and others) and examinations of resin and nectar would help to improve the realism of these tests. If pollen is the only source of bee exposure, then improved gene constructs that preferentially target expression to other plant tissues could reduce bee exposure to negligible levels.

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Résumé – Effets de l'ingestion d'une protéine liée à la biotine sur les adultes et les larves d'abeilles domestiques. Les propriétés insecticides des protéines liées à la biotine (BBP) ont été récemment exploitées pour produire des plantes transgéniques résistant aux ravageurs. Comme ces protéines ont un large spectre de toxicité pour les insectes, il est nécessaire d'évaluer l'impact potentiel sur des insectes non cibles tels que les abeilles domestiques (*Apis mellifera* L.). Dans cette étude on a fait ingérer à des adultes et des larves d'abeilles une BBP purifiée, l'avidine, et on en a déterminé les effets. Un régime comportant un dosage réaliste pour les larves a été mis au point en estimant la teneur en pollen de la nourriture larvaire pour des ruches placées à l'extérieur. L'avidine a été ajoutée au régime artificiel dans des quantités qui correspondaient à la présence de pollen transgénique exprimant l'avidine dans la nourriture larvaire. La concentration en pollen de la nourriture larvaire a été déterminée et le poids des grains de pollen individuels estimé. La nourriture larvaire des ruches à l'extérieur contenant en moyenne 2 895 ± 541 grains de pollen par mg de nourriture (de 0 à 21 969 grains). De nombreuses larves (probablement du 1er et 2e stade larvaire) pesant moins de 50 mg n'avaient pas de pollen détectable dans leur nourriture et aucune n'avait plus de 4 102 grains par mg de nourriture. Les larves plus grosses avaient entre 0 et 21 969 grains/mg de nourriture (Fig. 1). Des grains de pollen d'origine florale mélangée, qui avaient été conservés au froid, pesaient en moyenne 0,021 ± 0,003 µg (de 0,008 à 0,039 µg). Le poids des grains de pollen de tabac fraîchement récolté a été estimé à 0,011 µg. Puisque les effets d'une BBP sur un insecte peuvent être influencés par le niveau de biotine dans le régime, les niveaux de cette vitamine ont été déterminés dans la nourriture larvaire des cellules occupées par des larves jeunes et des larves plus âgées dans des ruches placées à l'extérieur et comparés avec un régime larvaire artificiel : 3,45 ± 0,73 µM, 3,14 ± 0,72 µM et 2,49 ± 0,49 µM respectivement (pas de différence significative). La biotine était présente dans les échantillons de pain d'abeilles au taux de 1,83 ± 0,08 µM et dans le pollen mélangé conservé au froid au taux de 1,85 ± 0,08 µM. Le test larve/avidine simulait une situation dans laquelle les abeilles recevaient 4 100 grains de pollen (pesant chacun 0,04 µg) par mg de nourriture durant les deux premiers jours de leur vie et 22 000 grains par mg de nourriture durant le reste de leur développement larvaire. De l'avidine purifiée a été ajoutée au régime artificiel à des concentrations correspondant à la quantité de pollen mentionnée ci-dessus et exprimant 0,4 ou 40 µg d'avidine. Toutes les abeilles ont été conservées jusqu'à leur mort. On n'a pas observé de différence significative dans la survie des abeilles qui puisse être attribuée à l'addition d'avidine au régime larvaire (Fig. 3 ; Tab. I). L'avidine n'a affecté ni le début de la défécation larvaire, ni l'émergence des adultes (Fig. 2). Dans une seconde expérience, des abeilles adultes fraîchement écloses ont été nourries avec un

mélange de pollen et de candi auquel de l'avidine purifiée avait été ajoutée aux doses correspondant à l'expression dans le pollen de 6,7 ou 20 µM d'avidine. Là encore aucune différence significative n'a pu être attribuée à l'avidine, que ce soit dans la quantité du mélange pollen-candi consommé (Fig. 4) ou dans leur survie (Fig. 5). Les résultats suggèrent que les plantes transgéniques exprimant de l'avidine dans le pollen à des doses équivalentes ou supérieures à celles enregistrées dans les feuilles ne sont toxiques ni pour les larves ni pour les abeilles adultes.

***Apis mellifera* / protéine liée à la biotine / avidine / plante transgénique**

Zusammenfassung – Auswirkung der Aufnahme von Biotin bindenden Eiweißen auf adulte Honigbienen und ihre Larven. Die insektizide Eigenschaft der Biotin bindenden Eiweiße (BBPs) wurden in letzter Zeit genutzt, um krankheitsresistente, transgene Pflanzen zu erzeugen. Da diese Proteine ein weites Spektrum von Insektengiftigkeit aufweisen, muss ihre Auswirkung auf Nicht-Zielinsekten untersucht werden, wie z.B. die Honigbienen. In dieser Arbeit wird die Wirkung der Fütterung von reinem BBP, Avidin, auf Larven und auf adulte Honigbienen bestimmt. Um eine realistische Verabreichungsdosis zu entwickeln, wurde der Pollengehalt der Brutnahrung im Feldversuch bestimmt. Avidin wurde einer künstlichen Diät in einer Menge zugefügt, die dem Vorhandensein von transgenem Avidin erzeugendem Pollen in der Brutnahrung entsprechen könnte. Die Pollenkonzentration im Larvenfutter wurde bestimmt und das Gewicht der einzelnen Pollenkörner wurde abgeschätzt. Das Larvenfutter der Völker im Feld enthielt im Mittel 2 895 ± 541 Pollenkörner pro mg Futter (Bereich von 0 bis 21 969). Bei vielen Larven, die weniger als 50 mg (wahrscheinlich 1. oder 2. Larvenstadium) wogen, wurden keine Pollen im Futter nachgewiesen, keine dieser Larve hatte mehr als 4 102 Körner pro mg Futter. Größere Larven hatten zwischen 0 und 21 969 Körner pro mg (Abb. 1). Eine Mischung aus unterschiedlichen Pollenkörnern, die kühl gelagert waren, hatten ein geschätztes mittleres Gewicht von 0,021 ± 0,003 µg (Bereich von 0,008–0,039 µg). Das Gewicht von frisch gesammelten Pollenkörnern von Tabak wurde jeweils auf 0,011 µg geschätzt. Da die Wirkung von BBP auf Insekten durch die Menge von Biotin in der Diät beeinflusst sein könnte, wurde die Menge dieses Vitamins im Larvenfutter aus Zellen mit jungen bzw. alten Larven in den Kontrollvölkern bestimmt und mit der künstlichen Larvendiät verglichen (3,45 ± 0,73 µM, 3,14 ± 0,72 µM bzw. 2,49 ± 0,49 µM, kein signifikanter Unterschied). Der Anteil des Biotin betrug 1,83 ± 0,36 µM in Bienenbrotproben aus Völkern und 1,85 ± 0,08 µM in gemischtem, kühl gelagertem Pollen. In den Futterversuchen entsprach das Verhältnis Larven/Avidin einer Situation, in der Bienen in den ersten beiden Larventagen 4 100 Pollenkörner (jedes wog 0,04 µg) per mg Futter erhielten und 22 000 Körner während des Restes der Larvenentwicklung. Gereinigtes Avidin wurde der künstlichen Diät in Konzentrationen zugefügt, die der Menge der oben berechneten Pollen entsprach, mit einer angenommenen Exprimierung von 0 µM, 4 µM oder 40 µM Avidin. Alle Bienen wurden bis zu ihrem Tod gehalten. Es gab keine signifikanten Unterschiede in der Lebensdauer der Bienen, die auf das dem Larvenfutter zugefügte Avidin zurückgeführt werden konnten (Abb. 3 und Tab. I). Der Beginn des larvalen Abkotens und des Schlupfs der Adulten blieb unbeeinflusst vom Avidin (Abb. 2).
In einem 2. Versuch wurde frisch geschlüpften Bienen eine Futterteigmischung mit Pollen und reinem Avidin angeboten, dessen Menge einer Exprimierung von Avidin im Pollen von 0 µM, 6,7 µM und 20 µM entsprach. Auch hier konnten keine signifikanten Unterschiede gefunden werden, weder bei der Menge des aufgenommenen, mit Avidin vermischten Futters noch bei der Lebensdauer (Abb. 5). Auf Grund dieser Ergebnisse wird angenommen, dass transgene Pflanzen, die Avidin im Pollen in gleicher oder sogar höherer Menge als in den Blättern exprimieren, weder für die Larven noch für die adulten Honigbienen giftig sind.

***Apis mellifera* / Biotin bindendes Eiweiß / Avidin / transgene Pflanze**

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